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Nanotechnologies — Considerations for performing toxicokinetic studies with nanomaterials

Nanotechnologies - Considérations pour réaliser des études toxico cinétiques de nanomatériaux

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Contents

Page

Foreword

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This document was prepared by Technical Committee ISO/TC 229, *Nanotechnologies.*

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Introduction

Nanomaterials (NMs) are a family of chemicals that, like any other chemicals, can exert a range of toxicities. Toxicokinetics can support the safety evaluation of compounds including NMs by identifying potential target organs, and especially for NMs, the potential for persistence in organs (including cellular uptake and compartmentalization). Also, toxicokinetic information can be used to evaluate if a NM behaves differently from a similar NM or bulk material with the same chemical composition, e.g. with regard to barrier penetration. As for all studies with NMs, a proper characterization of the NM dispersions or aerosols used in the toxicokinetic studies is essential.

Importance of toxicokinetic information for risk assessment (of nanomaterials)

Toxicokinetics describes the absorption, distribution, metabolism and excretion (ADME) of foreign compounds in the body with time. It links the external exposure with the internal dose and is thus a key aspect for toxicity. If a NM is absorbed by the body through any of the potential exposure routes (oral, respiratory, dermal) it can enter into the blood or lymph circulation. Subsequent distribution to internal organs determines potential target tissues and potential toxicity. Alternatively, NMs can be intravenously administered (e.g. as nanomedicine) thus directly entering the blood circulation, potentially resulting in wide spread tissue distribution. Toxicokinetics therefore aids in the design of targeted toxicity studies and in identifying potential target organs and can thus also provide relevant information for justification or waiving of toxicity studies. In addition, toxicokinetic information can be useful as basis for grouping and read-across of NMs. Risk assessments based on internal concentrations, determined using toxicokinetic information, can be more realistic than risk assessments based on external doses, as nanoparticles (NPs) can show specific tissue distribution and accumulation. Toxicokinetic studies can be used to build toxicokinetic models, especially physiologically based pharmacokinetic (PBPK) models, which then can be used to extrapolate experimental toxicity data to other species, tissues, exposure routes, exposure durations and doses. Due to the accumulation of some NPs, the ability to extrapolate to longer exposure durations is of special importance for NMs.

Why a technical report specifically for nanomaterials?

A considerable body of published literature, including many national and international guidelines, exists on the use of toxicokinetic methods to study the fate of chemicals in the body. In addition, OECD Test Guideline (TG) 417 on Toxicokinetics (latest update dated 2010) gives an extensive description for evaluation of the toxicokinetic profile of chemicals but excludes NMs specifically. ISO 10993-16:2017 *Biological evaluation of medical devices — Part 16: Toxicokinetic study design for degradation products and leachables*, provides an overview for toxicokinetic studies for leachables of medical devices. Furthermore, the European Medicines Agency's ICH S3A (Toxicokinetics: A Guidance for Assessing Systemic Exposure in Toxicology Studies) and ICH S3B (Pharmacokinetics: Repeated Dose Tissue Distribution Studies) give guidance on the design and conduct of toxicokinetic studies to assist in the development of new drugs.

Guidelines also exist on toxicokinetic modelling, especially the development and application of physiologically-based pharmacokinetic (PBPK) models. For example, the United States Food and Drug Administration's Draft Physiologically Based Pharmacokinetic Analyses — Format and Content Guidance for Industry, provides the standard content and format of PBPK study reports while the United States Environmental Protection Agency's Approaches for the Application of Physiologically Based Pharmacokinetic (PBPK) Models and Supporting Data in Risk Assessment, addresses the application and evaluation of PBPK models for risk assessment purposes. The European Medicines Agency (EMA) has published a "Guideline on the qualification and reporting of physiologically based pharmacokinetic (PBPK) modelling and simulation" in 2016[1]. WHO has published the "Characterization and application of physiologically based pharmacokinetic models in risk assessment"[2].

As stated, the current OECD toxicokinetics TG 417 explicitly states that the guideline is not intended for the testing of NMs[3], as the toxicokinetics of NMs are different from dissolved ions/molecules and large particles. This was confirmed in a report on preliminary review of OECD Test Guidelines for their applicability to NMs[4]. Additionally, the PBPK models described in the current and mentioned guidance documents are not suitable for NMs, as the processes governing the distribution of NPs is different from

those of the dissolved (molecular/ionic) substances addressed by the current guidance documents (e.g. Reference [5]).

New guidelines or specific additions to existing guidelines about the case of NMs are thus necessary. A review of the current knowledge on the specific toxicokinetic characteristics of NMs and the issues around toxicokinetic testing is a practical preparative step to ensure the best possible understanding of testing needed to obtain relevant information on toxicokinetics of NMs.

How are nanomaterials different from dissolved ions/molecules and large particles?

Nanomaterials (NMs) present a unique family of chemicals that, by their particulate nature and reduction in size, acquire specific physical chemical properties not present for their bulk or soluble counterparts, that might or might not be accompanied by specific toxicity as discussed previously in many reports (e.g. References [6], [7], [8], [9], [10]).

Toxicokinetics of NPs is of special interest because, in comparison to larger sized particles, the small size of NPs could enable an increased rate of translocation beyond the portal of entry, to the lymphatic fluid and blood circulation, from where they can reach potentially all internal organs[11]. In addition, smaller sized NPs can show a more widespread organ distribution than larger sized particles[12]. For the same reason, transport across barriers such as the blood-brain barrier and placenta can occur (e.g. References [13] and [14]).

Other notable differences between the toxicokinetic behaviour of dissolved molecular/ionic substances and NMs can be understood within the context of the principles that govern the absorption, distribution, metabolism and excretion (ADME) of a substance. For dissolved molecular/ionic substances, toxicokinetics is driven by 1) passive transport, which includes simple diffusion and filtration or 2) special transport, which includes active transport, carrier-mediated transporter systems and facilitated diffusion through cellular membranes, enzymatic metabolism and passive or active excretion. For NMs, toxicokinetics involves aggregation, agglomeration, protein corona formation, active cellular uptake, distribution through macrophages, and for certain NMs degradation, and excretion[15]. In addition, the surface chemistry/composition affects the toxicokinetics of NPs by its potential of binding a variety of biomolecules on the surface (also designated the "protein" corona). As excretion is often limited, bioaccumulation can occur similar to other poorly metabolized molecules. Thus, the requirements for the testing and modelling of the toxicokinetics of NMs can differ significantly from those identified for dissolved substances. In this respect, especially the potential for accumulation and persistence in organs needs to be evaluated, for example in repeated dose and prolonged toxicokinetic studies.

Nanotechnologies — Considerations for performing toxicokinetic studies with nanomaterials

1 Scope

This document describes the background and principles for toxicokinetic studies relevant for nanomaterials.

Annex A shows the definitions for terminology with respect to toxicokinetics as used in OECD TG 417:2010.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

For the purposes of this document, the terms and definitions given in the ISO 80004 series Nanotechnologies Vocabulary and the following apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at http://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

3.1
agglomerate
collection of weakly or medium strongly bound *particles* (3.12) where the resulting external surface area is similar to the sum of the surface areas of the individual components

Note 1 to entry: The forces holding an agglomerate together are weak forces, for example van der Waals forces or simple physical entanglement.

Note 2 to entry: Agglomerates are also termed secondary particles and the original source particles are termed primary particles.

[SOURCE: ISO 26824:2013, 1.2]

3.2
aggregate
particle (3.12) comprising strongly bonded or fused particles where the resulting external surface area is significantly smaller than the sum of surface areas of the individual components

Note 1 to entry: The forces holding an aggregate together are strong forces, for example covalent or ionic bonds, or those resulting from sintering or complex physical entanglement, or otherwise combined former primary particles.

Note 2 to entry: Aggregates are also termed secondary particles and the original source particles are termed primary particles.

[SOURCE: ISO 26824:2013, 1.3, modified — Note 1 adapted.]

3.3
nanoscale
length range approximately from 1 nm to 100 nm

Note 1 to entry: Properties that are not extrapolations from larger sizes are predominantly exhibited in this length range.

[SOURCE: ISO/TS 80004-1: 2015, 2.1]

3.4
nanotechnology
application of scientific knowledge to manipulate and control matter predominantly in the *nanoscale* (3.3) to make use of size- and structure-dependent properties and phenomena distinct from those associated with individual atoms or molecules, or extrapolation from larger sizes of the same material

Note 1 to entry: Manipulation and control includes material synthesis.

[SOURCE: ISO/TS 80004-1: 2015, 2.3]

3.5
nanomaterial
material with any external dimension in the *nanoscale* (3.3) or having internal structure or surface structure in the nanoscale

Note 1 to entry: This generic term is inclusive of *nano-object* (3.6) and *nanostructured material* (3.8).

Note 2 to entry: See also 3.6 to 3.11.

[SOURCE: ISO/TS 80004-1: 2015, 2.4]

3.6
nano-object
discrete piece of material with one, two or three external dimensions in the *nanoscale* (3.3)

Note 1 to entry: The second and third external dimensions are orthogonal to the first dimension and to each other.

[SOURCE: ISO/TS 80004-1: 2015, 2.5]

3.7
nanostructure
composition of inter-related constituent parts in which one or more of those parts is a *nanoscale* (3.3) region

Note 1 to entry: A region is defined by a boundary representing a discontinuity in properties.

[SOURCE: ISO/TS 80004-1: 2015, 2.6]

3.8
nanostructured material
material having internal *nanostructure* (3.7) or surface nanostructure

Note 1 to entry: This definition does not exclude the possibility for a *nano-object* (3.6) to have internal structure or surface structure. If external dimension(s) are in the *nanoscale* (3.3), the term nano-object is recommended.

[SOURCE: ISO/TS 80004-1: 2015, 2.7]

3.9
nanoparticle
nano-object (3.6) with all external dimensions in the *nanoscale* (3.3) where the lengths of the longest and the shortest axes of the nano-object do not differ significantly

Note 1 to entry: If the dimensions differ significantly (typically by more than 3 times), terms such as *nanofibre* (ISO/TS 80004-2:2017, 4.5) or *nanoplate* (ISO/TS 80004-2:2017 4.6) may be preferred to the term nanoparticle.

[SOURCE: ISO/TS 80004-2:2017, 4.4, modified — Note 1 to entry has been changed for clarification.]

3.12
particle
minute piece of matter with defined physical boundaries

Note 1 to entry: A physical boundary can also be described as an interface.

Note 2 to entry: A particle can move as a unit.

Note 3 to entry: This general particle definition applies to *nano-objects* (3.6).

[SOURCE: ISO 26824:2013, 1.1]

3.13
substance
single chemical element or compound, or a complex structure of compounds

[SOURCE: ISO 10993-9:2009, 3.6]

4 Abbreviations

AAS	Atomic Absorption Spectrometry
ADME	Absorption, Distribution, Metabolism, Excretion
AUC	Area under the Curve
BALF	Bronchoalveolar lavage fluid
ICP-MS	Inductively Coupled Plasma – Mass Spectrometry
IV	Intravenous
IVIVE	in vitro in vivo extrapolation
MPS	mononuclear phagocytic system
MWCNT	Multi Walled Carbon Nanotubes
NM(s)	Nanomaterial(s)
NP(s)	Nanoparticle(s)
PBPK	Physiologically Based Pharmacokinetic (model)
SSA	Specific Surface Area
TG	Test Guideline

5 Importance of toxicokinetic information for risk assessment of nanomaterials

5.1 General

Toxicokinetic studies are important to obtain insight in the toxicologically relevant target organs that can be considered more closely in the safety evaluation and risk assessment of NMs and/or NPs. Furthermore, information might be obtained on relevant exposure durations (e.g. acute, chronic) to be applied in toxicity studies based on the persistence of the NP over time. Finally, such information is essential to enable more reliable extrapolations over species, time and exposure routes and can be used for grouping, read-across and waiving.

5.2 Possible use of toxicokinetic information

For dissolved substances, legislation differs in the requirement for providing kinetic information, also between countries, but most often this information is not required by legislation[16]. However, toxicokinetic knowledge is essential for various purposes in the current risk assessment approach based on animal tests:

— to predict systemic exposure and internal tissue dose (correlate given dose with target dose);

— to know whether a test, such as a genotoxicity test in bone marrow or sperm, is relevant (does the substance reach these tissues?);

— to perform route-to-route extrapolation (see e.g. Reference [17]);

— to perform high-to-low-dose extrapolation or to select appropriate doses (see e.g. Reference [18] and [19]);

— to verify human relevance of test results from animals (i.e. perform interspecies extrapolation; e.g. Reference [20]);

— to enable extrapolation in time for accumulating substances, as animal tests do not cover an entire human lifetime, while accumulation can lead to increases in concentration in a tissue that continues lifelong (e.g. Reference [21]).

When avoiding animal tests as much as possible and performing a risk assessment based mostly on *in vitro* test results, as envisioned by the 3Rs principle[22], kinetic information becomes even more essential. *In vitro* tests do not provide for the totality of the toxicokinetics of a whole body, as animals do: the absorption in the intestines, for example, is not included in an *in vitro* test with liver cells. Thus, *in vitro* test results need to be supplemented with kinetic information using kinetic models, in a process named *in vitro in vivo* extrapolation (IVIVE).

In addition, toxicokinetic information provides insight into potential target organs and organ burden that might ultimately result in toxicity. This allows for improved selection and design of hazard studies, e.g. waiving a certain systemic study if absorption and accumulation of the substances are known not to occur, or adding additional analyses to a study that are relevant to identified target organs.

These considerations are valid for both NMs and soluble substances. Specific for non-degradable NMs is that there is a higher potential for accumulation. In the case of accumulation, determination of the kinetics is of greater importance for the correct estimation of a health risk, as an extrapolation in time needs to be made. This is valid for accumulating NMs just as much as it is for accumulating substances. Internal (or target tissue) concentrations are therefore better dose metrics for risk assessment purposes than external doses.

Specific for NMs is also that they have a distinct distribution pattern, with high proportions in organs of the mononuclear phagocytic system (MPS) notably in the liver and spleen. Such information can, for example, warrant special attention for potential effects on liver and spleen cell populations[21][23].

Due to the many forms in which NMs can occur or be produced, of which testing all would require a large amount of resources, grouping is of high interest for NMs. Recent papers on possibilities for grouping of NMs describe kinetic parameters as essential pieces of information on which to base group formation and justification: degradation (including dissolution), distribution and potential bioaccumulation or persistence and distribution[24][25][26]. Dissolution is actually a physico-chemical parameter that also is dependent of the local environment (e.g. water, buffer or (simulated) body fluids), but can also be seen as a kinetic parameter. The rate of dissolution/degradation provides insight in the toxicokinetic behaviour of a NM. Until dissolution occurs, the kinetics of NMs are governed by the particulate nature of the NMs, whereas after dissolution the (dissolved) ions or molecules determine the toxicokinetics. Distribution studies are needed to assess if and to which extent the different NMs show distribution to the same target organs, as part of a scientific justification for grouping, and to assess if the same hazards can be considered. Accumulation is a kinetic parameter, which is not measured directly, but is determined by all other (more basic) kinetic parameters, i.e. absorption, distribution, and elimination.

5.3 Key toxicokinetic issues for nanomaterials

The kinetic properties of a compound include the biodistribution, biodegradation and biopersistence and can be described by the time course for absorption, distribution, metabolism and excretion (ADME) of a compound in the body with time. Absorption, distribution, (metabolism), and excretion can be described as potentially sequential processes. The basic principles that are described in OECD 417[3] and ISO 10993-16:2017 provide a framework how to perform toxicokinetic studies. An OECD Expert meeting, Toxicokinetics of Manufactured Nanomaterials, identified issues for toxicokinetics for NMs and discussed how to address them[27].

The absorption of current NMs/nano-objects after oral exposure is commonly very low, in the order of 1 % and less[17][28].

Another major difference between the toxicokinetics of dissolved substances and NMs is that the tissue distribution for dissolved substances is concentration dependent (i.e. the difference in concentration in the circulation/blood and the organ determines the organ uptake), and that an equilibrium is generally obtained between blood and organ concentration. In contrast, NMs/NPs are rapidly removed from the systemic circulation by cells of the mononuclear phagocytic system (MPS) as indicated by the observed distribution of a major fraction of an injected dose into spleen and liver[12][17][29]. However, also granulocytes are able to take up NPs[30]. This implies that plasma is usually not a suitable media to monitor NP exposure and plasma kinetic parameters such as plasma area under the curve (AUC) are generally not relevant. In addition, PBPK-models for NPs need to be based on blood flow and the uptake of the NPs by macrophages (e.g. References [5], [31] and [32]), or need to consider specific targeting by ligands as components of an NM for drug targeting[33][34], instead of equilibrium partitioning.

Regarding the metabolism, biotransformation or degradation might be a more appropriate term given the uncertainty associated with the occurrence of enzymatic metabolism for many NMs (e.g. for inorganic NMs such as the metal and metal oxides). However, organic NMs can be metabolized. The dissolution of a NM can also be seen as a more general process that transforms NMs, and is thereby similar to metabolism.

Excretion of systemically available NMs is possible through breast milk[35], urine and bile[13], but seemingly not for all types of NPs. For some NPs (e.g. TiO_2), the only elimination route (besides breast milk) seems to be dissolution, which renders insoluble NMs very persistent and accumulative.

Thus, even though kinetic information in general is just as important for molecular substances as for NMs (see 5.1), the type of kinetic information that is necessary differs and other issues arise when testing for toxicokinetic properties. Key kinetic parameters for NMs are:

— degradation, which is determined mostly by the dissolution rate in the various physiologically relevant surroundings (incl. in macrophages) (elaborated on in 6.1);

— absorption (i.e. translocation over the external barriers, dependent on the exposure route) (elaborated on in chapter 9);

— uptake by macrophages/granulocytes or by monocytes in tissues (as a very new parameter, feasibility yet unknown);

— elimination rate from the tissues (elaborated on in chapter 12).

The latter can, together with physiological information on macrophage content of tissues, help determine the potential uptake rate into tissues. Ultimately these key parameters determine the tissue distribution of the NM and indicate the target organs potentially at risk for toxic effects.

6 Factors influencing the toxicokinetics of nanomaterials

6.1 Dissolution rate

A major factor for the induction of an adverse (toxic) effect by NMs is considered to be related to the presence or release of free nano-objects, ions, molecules or components from the individual NM. In

this respect, the dissolution, or rather the dissolution rate, of the NM can be considered crucial for risk assessment. If a NM has been completely dissolved before absorption, the classical risk assessment of the dissolved chemical/molecules can be applied (i.e. no special NM considerations are applicable[36][37]).

NM dissolution rates have been found to be extremely sensitive to variables of the experimental testing protocol, e.g. NM dispersion procedure, primary and agglomerate/aggregate size distributions, temperature, pH, composition of the test medium, hydrodynamic conditions (stirring, etc.). This sensitivity is significantly larger than with dissolved substances. Furthermore, there is still no consensus on which is the most suitable combination of solid-liquid separation step (ultrafiltration, ultracentrifugation, etc.) and elemental analysis technique (atomic spectrometry, voltammetry, etc.), nor which dissolved fraction (free ions, low MW dissolved complexes, metal bound to macromolecules, etc.) is the most relevant for toxicology purposes. Therefore, NM dissolution rate in physiologically relevant media seems to still be an ill-defined endpoint from a regulatory point of view. Further development and standardization of test methods for dissolution rate is therefore highly necessary (ISO/TR 19057)[38].

The dissolution rate of a given NM in humans varies with type of body fluid e.g. through differences in pH of these fluids. It is therefore relevant to determine the dissolution rate in a representative set of media, which mimic the relevant body fluids. Relevant body fluids are not only saliva, lung mucus, gastric juice, intestinal fluid and plasma, but also lysosomal fluid, as NMs are known to end up in lysosomes of macrophages[37]. As an example, NiO nanowire-like particles were 100 % dissolved within 24 h when mixed with artificial lysosomal fluid, while they dissolved only minimally (3,5 % to 6,5 %) in water, saline and artificial interstitial lung fluid (Gamble's solution) at 216 h. Spherical NiO NPs were only 12 % and 35 % dissolved after 216 h when mixed with artificial lysosomal fluid, and the largest, irregular-shaped NiO NPs hardly dissolved in any solution indicating an effect of shape[39]. In this case, the nanowire like particles are eliminated within 24 h. Both in the case of the nanowirelike particles and the nanospheres, NPs and ions can be present during the first 24 h, but at a different ratio (that is changing in time), impacting the risk assessment.

6.2 Physical chemical properties determinant for toxicokinetic behavior

Several distinct factors influence the kinetics of ENM (apart from those that also influence the kinetics of molecular substances)[13][40][41][42][43]:

— the size (primary particle and agglomeration/aggregation) of NM;

— the surface charge of NM;

— the morphology/shape (e.g. the aspect ratio in case of fibres);

— protein binding to NM;

— surface chemistry (e.g. coatings, hydrophobicity).

Both the size and surface charge have shown to affect the composition and density of proteins attached to NPs[44].

As for the dissolution rate, these physical-chemical properties might change in different environments, e.g. as pristine material, in dosing medium, body fluids, and in tissues. Therefore, physical-chemical characterization may need to be determined at various stages of the toxicokinetic testing.

It is still difficult to analyse the relationship between phys-chem properties of NMs and their toxicokinetic behaviour, as there are few studies systematically studying these relationships by varying one property at a time. In addition, the quality of the studies is not always sufficient, especially those from the beginning of the research on NMs, when there was still too little knowledge to ensure certain quality.

For every study performed with NMs, including toxicokinetic studies, knowledge on the physicochemical parameters (e.g. size, agglomeration/aggregation, morphology, degradation/dissolution, surface charge, surface chemistry) of the NM dispersion or aerosol evaluated needs to be available. For information on

the determination of various physicochemical parameters a number of ISO documents are available (for overview see ISO TR 18196:2016).

Size

In many studies, it was observed that the smaller sized NM resulted in a more widespread biodistribution, i.e. to other tissues, compared with larger-sized NM. For example, when comparing the size of Au-NPs, it was reported that the smallest NPs (i.e. 10 nm) showed the most widespread organ distribution after intravenous administration[12]. Poly(amidoamine) PAMAM dendrimers of 5 nm sized particles showed a more favourable distribution to tumors in mice compared to the 11 nm and 22 nm particles, which was suggested to be due to less immune recognition and less organ-specific binding[45]. Conflicting results have been obtained on the effect of size on the pattern of distribution: For Ag-NPs, the 20 nm particles distributed mainly to liver after IV injection, followed by kidneys and spleen, whereas the larger particles (80 nm and 110 nm) distributed mainly to spleen followed by liver and lung. In the other organs evaluated, no major differences between the sizes were observed[29]. In another study, however, also for Ag-NPs after intravenous administration, all particle sizes investigated (10 nm, 40 nm, and 100 nm), regardless of their coating, showed the highest silver concentrations in the spleen and liver, followed by lung, kidney, and brain[46].

There are also reports that smaller NM lead to higher organ concentrations, indicating higher tissue specific absorption. For example, silver concentrations were significantly higher in the spleen, lung, kidney, brain, and blood of mice treated with 10 nm Ag NPs than those treated with larger particles. This finding correlated with relevant adverse effects (midzonal hepatocellular necrosis, gall bladder haemorrhage) observed in the mice treated with 10 nm Ag NPs, while lesions observed in mice treated with 40 nm and 100 nm Ag NPs lesions were milder or negligible, respectively[46]. Following inhalation exposure, there was no size dependent retention observed in the lung. All sizes of NPs investigated (10 nm, 15 nm, 35 nm and 75 nm of iridium-192 NPs) showed similar retention times[47]. There was a slow long-term clearance of iridium from the rat lung (i.e. retention half-times of several hundred days). There was a low translocation in the body (maximum 0,4 % of the lung burden). However, organ distribution to the liver and spleen did show a marked difference between the 10 nm and 15 nm size on the one hand and 35 nm and 75 nm on the other hand with high levels for the smaller NPs. Kreyling et al[48] also demonstrated that smaller iridium and carbon NPs showed a higher translocation into the body following inhalation[48].

A complicating factor in systematic evaluation of the influence of the NP size on toxicokinetics, is that the other properties (e.g. shape, surface composition etc.) need to be kept similar among the tested particles.

There seem to be optimal size ranges for tissue uptake, which differ with the organ and the cell type *in vitro*, respectively. For agglomerated NPs (>0,5 µm), uptake by macrophages is expected to be the major internalization pathway while smaller particles are primarily processed by endocytotic pathways[13]. For gold NPs, it has been found that the optimum size for uptake in human breast cancer SK-BR-3 cells is 25 nm to 50 nm[49].

Another aspect of size is the fact that NPs can form superstructures based on the primary particles as potential building blocks in the formation of agglomerates (i.e. structures that are formed by weak Van Der Waals forces) and aggregates (i.e. structures formed by strong molecular binding forces). For Au NPs with a primary particle size of 5 nm to 8 nm, these structures can have sizes ranging from 40 nm up to 2 000 nm[50]. A confounding effect was noted with the intravenous dosing of the Au nanostructures with a considerable amount of aggregates remaining in the administration syringes. Although such losses to labware have been reported more often and might not be specific for aggregates, it is possible that aggregate losses occur more readily due to their faster sedimentation. Aggregation can change the potential to accumulate, leading to size-dependent differences in distribution. The difference in distribution is seen most dramatically in the lung with the aggregates showing significantly higher accumulation compared with the primary particles. Aggregates also accumulated significantly more in the heart[50].

Surface properties, including charge

The surface and especially the coating of the NPs can also have an effect on NP distribution, partly through the modification of the surface charge. As different cells can have a different membrane charge, which is dependent on their redox state, NPs with a certain surface charge will not be taken up by all cells with equal efficiency[51]. For dendrimer-coated NPs, in addition to the effect of their size as described above, the organ distribution pattern was also affected by the surface charge, as positively charged dendrimers showed higher distribution to the kidney, whereas neutral non-charged and negatively charged 5 nm dendrimers tended to be preferentially distributed to liver and spleen[45]. Similar observations were made for gold/dendrimer composite nanodevices[52]. For quantum dots (QDs) biodistribution studies demonstrated that negative and neutral CdSe/ZnS QDs preferentially distributed in the liver and the spleen, whereas positive QDs mainly deposited in the kidney and in the brain[53]. From these studies it might be concluded that a positive surface charge would favour migration to the kidneys.

It is also well known that coating NPs or nanorods with polyethyleneglycol (PEG) affects the half-life in the blood[31][54][55]. This is probably more a steric effect than a charge effect. This has been demonstrated for example for the PEGylation of Au nanorods, Au NPs and organic nanotubes[55][56][57][58]. Higher PEG grafting levels were advantageous for MPS avoidance, for enhancing permeability and retention (EPR) in tumor sites, and for suppression of aggregation of the gold nanorods in the circulation[56]. For the PEG-Au-NPs, intravenous injection of 4 nm and 13 nm NPs showed a prolonged circulation time up to 7 d[57]. These kinetic trends were well correlated with tissue distribution patterns, particularly in liver, spleen, and mesenteric lymph node. PEGylated particles bind very few proteins, avoid uptake by the MPS, and therefore circulate longer in the blood[59][60]. Also for citrate-ligand-capped 10 nm Au NPs and PEGylated 10 nm Au NPs a difference was observed for distribution to breast milk after intravenous administration to lactating mice with the PEGylated Au NPs showing a higher and more prolonged presence in breast milk[61].

For three different Ag NP sizes (10 nm, 40 nm, 100 nm) there was no difference in the distribution between Ag NPs coated with citrate-or polyvinylpyrrolidone[46].

Several ISO documents are available describing methods for the surface characterization of NMs and NPs, including ISO/TR 13014:2012, ISO/TS 14101:2012, ISO/TR 14187:2011, and ISO 20579-4:2018. For consistent surface chemistry also issues regarding reproducibility of the NPs themselves are important to consider due to their inherent variability. An appropriate set of characterization methods consistently applied at critical times can be useful tools to assess and identify reproducibility and sources of variability[62].

Protein corona

After contact with any biological environment the NPs will immediately be coated with biomolecules as present in the surrounding biological matrix[59]. This layer of mostly proteins is generally considered as the “protein corona”. With as many as 3 700 proteins expected in the complete plasma proteome, and even other proteins present in other fluids, it is clear that the composition of the protein corona around a NP can vary enormously and does affect toxicokinetics of NP and the recognition by cells[59][63][64][65]. Even serum might not be the perfect model for the *in vivo* blood environment, because it misses the blood coagulation factors[44].

An important identified effect of the protein corona, which depends on its composition is, for example, the stabilization of a NP. Albumin in water or Dulbecco’s Modified Eagle Medium (DMEM) has shown to stabilize many NMs, protecting against agglomeration. Bronchoalveolar lavage fluid induces the opposite effect, increasing agglomeration.

A second important effect of the protein corona is that some proteins (the opsonins) lead to recognition by macrophages. Walkey et al.[44] found that the most active promotors of cell association (which can probably be regarded as opsonins) were proteins known to be involved in binding to hyaluronan, a major component of cell surfaces. Opsonisation therefore often causes fast uptake by macrophages, leading to a quick decrease of NPs in the circulation[59][66], fast transfer to and concentration in organs of the MPS [previously called the reticuloendothelial system (RES)], such as the liver and spleen. In contrast, dysopsonins (albumin, apolipoprotein A-I, A-IV, C-III, and H) reduce the affinity of NMs to the MPS. As opposed to the action of albumin, its fetal analog fetuin led to uptake of 50 nm polystyrene nanospheres by liver macrophages (Kupffer cells)[13].

Walkey et al.[44] found that the proportions of the serum proteins in the corona does not reflect the relative abundance of the proteins in serum itself; the properties of the NMs thus influence the corona composition. It might be clear that the surface characteristics of NPs which favor or disfavor opsonization profoundly influence their toxicokinetics. A hydrophilic and neutral surface of NMs disfavors binding of opsonins. Walkley et al.[44] found that the cationic gold NPs gave a higher cell association than the anionic and neutral gold NPs of the same size (all including their protein coronas). In contrast, there were few differences in the corona between several particles incubated with BALF (bronchoalveolar lavage fluid), although possibly also some cellular and serum proteins were detected due to agitation of the lung tissue due to BALF harvesting[67]. The BALF used was obtained from patients with pulmonary alveolar proteinosis which is rich in lipids and surfactant associated proteins.

It has also been found that protein coronas are dynamic, changing in composition with time. At first contact, the proteins present most abundantly in the particle environment bind to the particle, which are replaced in time by proteins for which the bond is more favourable energetically[68][69]. The most strongly bound proteins form the so called “hard” corona, while the proteins that have a dynamic exchange with their environment are designated the “soft” corona[68]. The effect of the protein corona on biological processes is yet unknown. However, the protein corona itself is composed of endogenous biomolecules that could contain binding motifs which can be recognised by cells[65].

The relative importance of the protein corona composition was shown by modelling work of Walkley et al.[44] The protein corona fingerprint of a library of 105 surface modified gold NPs was more predictive of (alveolar) A549 cell association than a model combining the information on core size (measured by TEM), dielectric environment (measured by AS), hydrodynamic diameter (measured by DLS) and the zeta potential. Also, the fingerprint was more predictive than the total amount of protein absorbed, indicating the importance of the protein corona composition. Interestingly, the model relating the protein corona fingerprint of gold NPs to their cell association could not predict the cell association from the protein corona fingerprint of silver NPs with some of the same surface ligands as used for the gold NPs. Clearly, the NP core itself has some unknown effect on the cell association. It was found that the core material (silver or gold) had a larger effect on the protein corona composition (larger difference in composition) than the ligands attached to the NP surface did. Probably, the properties of the core NP determine the density, arrangement, and orientation of the ligands, influencing the attachment of proteins[44].

Aggarwal et al.[59] provide a good overview on the various aspects of the protein corona and coating of NPs including the effects of the protein corona on biodistribution of the NPs.

This effect of protein corona composition raises the question whether this composition is different in the typical species used in animal tests, as for example rats are known to have higher levels of alpha-fetoprotein in serum protein than humans[70]. Possibly, this could lead to interspecies differences in toxicokinetics of NPs. When performing *in vitro* tests, the protein corona composition might be even further distant from the composition in humans, as culture medium has a different composition than the different body fluids. Some reassurance that the composition of the environment does not matter too much is obtained from the finding of Walkley et al.[44] that the corona composition does not reflect the proportions in the serum to which the NPs were exposed. Nevertheless, until certainty is provided that the protein composition of the surroundings does not influence the composition of the corona on the NP, the protein corona needs to be considered when performing *in vitro* studies and for interspecies extrapolation. Alternatively, NPs could first be exposed to a physiologically-mimicking fluid before being added to the *in vitro* test.

Shape

For gold NPs, it has been found that spherical NPs were taken up more readily by MCF-7cells than rod-shaped particles[51]. In addition, a smaller amount of longer rod-shaped gold NPs were taken up than shorter gold NPs with similar surface charges. The lower uptake of (longer) rod-shaped particles seems to lead to a lower elimination from cells, as well, as a higher accumulation has been reported. When using antibody targeted to adhesion molecules and neovascular expression markers on endothelial cells, Au coated nanorods showed a higher specific and lower non-specific accumulation under microfluidic flow conditions that mimic the vasculature, when compared to their spherical counterparts[71]. Similar